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13. ABSTRACT (Maximum 200 Words)
Breast cancer can be a genetic disease passed from mother to daughter. BRCA1 is the gene that when mutated is responsible for half of inherited breast cancer cases and about 80% of the combined breast and ovarian cancer kindreds. Therefore, the function of the protein product of BRCA1, which is still unknown, must be extremely important in mammary and ovarian cells because when it is no longer there the cells become cancerous. This project aims to determine how the BRCA1 protein performs its protective function. We have set up a biochemical analysis of BRCA1, which should reveal key pathways regulated by BRCA1. Specifically, this project assays how BRCA1 directs the ubiquitination of cellular proteins, which influence the growth of the cell. In cells in which BRCA1 is mutated, perhaps the loss of the ubiquitination results in cancerous transformation.

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Annual Summary Report

Award Number DAMD17-02-1-0306, Predoctoral fellowship
PI: Lea Starita
Mentor: Jeffrey D. Parvin, MD, PhD

Introduction
I have made considerable progress on the research project that I outlined in the pre-doctoral DOD breast cancer research fellowship proposal. The proposal aimed to determine targets for the ubiquitin ligase activity of BRCA1. BRCA1 (**Br**east **Ca**ncer susceptibility gene **1**) is an important tumor suppressor that protects mammary cells from malignant transformation. Recently BRCA1 has been found to have an enzymatic function as an ubiquitin ligase. Ubiquitin ligases tag other proteins with a small peptide, ubiquitin, and these tagged proteins then signal for downstream events such as protein degradation or DNA repair. Determination of the targets for ubiquitination by BRCA1 will lead to a better understanding of how this protein performs its tumor suppressor function.

Body and key research outcomes
The specific aims for this project are as follows:
1. Identify targets for ubiquitination by BRCA1
    A. Biochemical experiments: use of purified BRCA1-containing complexes and ubiquitin activating and conjugating enzymes for in vitro reactions to determine which proteins are ubiquitinated by BRCA1.
    B. Biological experiments: use of HCC1937 cells, which contain a truncated form of BRCA1 to test the function of the carboxy-terminus in the regulation of ubiquitination.
2. Reconstitution of BRCA1 ubiquitination in a pathway in vitro.

Progress for aim 1A. We have developed a powerful in vitro ubiquitination system using purified ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme UbcH5c (E2), purified ubiquitin and BRCA1 co-purified with its heterodimeric binding partner BARD1 (**B**RCA1 **A**ssociated **R**ING **D**omain protein **1**). We have applied this in vitro system to an observation that we have made in which inhibition of BRCA1 in breast cells results in an amplification of centrosome number. We have determined that γ-tubulin associated with purified centrosomes is a target for BRCA1/BARD1 ubiquitination (see Figure 1). These results suggest that BRCA1 ubiquitination activity plays a role in the maintenance of centrosome number and integrity. These results have been submitted for publication, and we are currently adding an experiment and revising the manuscript prior to resubmission.

Using this in vitro ubiquitination system we have also determined that phosphorylated RNA pol II is also targeted by BRCA1 for ubiquitination. BRCA1 is known to co-purify

with RNA pol II. Interestingly, BRCA1 also plays a role in DNA damage repair suggesting that BRCA1 may be a genome surveillance protein scanning the genome as a passenger on RNA pol II.

Figure 1. γ-tubulin is ubiquitinated by BRCA1/BARD1. The centrosome and full length BRCA1/BARD1 were tested using in vitro ubiquitination reactions and analyzed by anti γ-tubulin western blot.

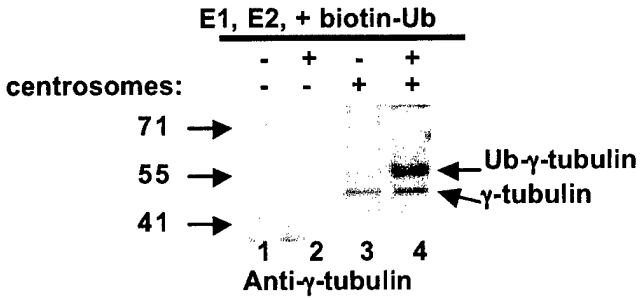
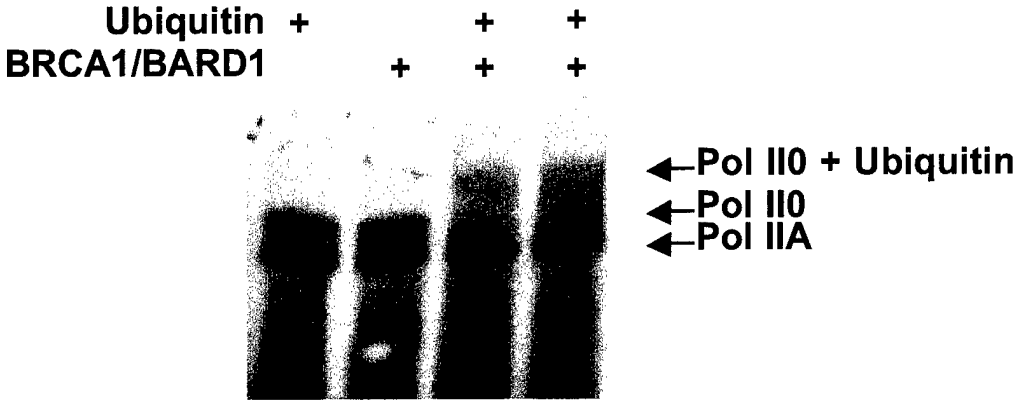


Figure 2. BRCA1/BARD1 ubiquitinates RNA pol II with specificity toward RNA pol II0. Purified RNA pol II is phosphorylated by TFIIH with γ-^{32}PATP prior to incubation with BRCA1/BARD1, His-E1, His-UbcH5c and ubiquitin. The specificity for the phosphorylated form of polymerase is suggestive that BRCA1 targets the transcriptionally engaged polymerase, consistent with our model.



Progress for aim 1B. We are using MCF10A or HS578T breast cell lines that are transfected with siRNA targeted against BRCA1 to confirm whether the targets for ubiquitination found in vitro are important in vivo. Assays for immunoprecipitating ubiquitinated proteins are still being optimized.

Progress for aim 2. Reconstitution of BRCA1 ubiquitination function in a pathway in vitro.

We have made some progress on this aim. We had proposed in the original application that BRCA1 was a component in a DNA damage sensing mechanism whereby DNA damage would be detected by transcription by RNA polymerase II as it

synthesized mRNA. BRCA1 and BARD1, present in the complex would then ubiquitinate the polymerase and signal for the initiation of damage repair. We have data, which support this model. First, there is the in vitro ubiquitination assay described above (Figure 2). These data are consistent with the pathway that we propose. The second line of evidence is that we have found that in cells, inhibition of transcription elongation signals to the cell that there is DNA damage. We are currently determining whether this pathway depends on functional BRCA1.

Reportable Outcomes: Publications resulting from DAMD17-02-1-0306
We anticipate resubmitting in the very near future the manuscript describing the BRCA1-mediated ubiquitination of centrosomes.

Review article:
Starita LM, Parvin JD. The multiple nuclear functions of BRCA1: transcription, ubiquitination, and DNA repair. **Current Opinion In Cell Biology** 2003; *15,* 345-350.

Conclusions
We have developed a highly active and highly specific BRCA1-dependent ubiquitination assay for in vitro assays. Using this novel experimental system, we have identified two substrates: centrosomes and RNA polymerase II. We have begun to characterize the biochemical pathway in which BRCA1-dependent ubiquitination activity links transcription of mRNA to the DNA damage response. This is outstanding progress in the first year of this fellowship towards achieving the goals of this project.

Appendix
See enclosed a review article supported in part from this fellowship.

CURRENT
OPINION
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The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair

Lea M Starita and Jeffrey D Parvin

Interest in BRCA1 stems from its role as a tumour suppressor in breast and ovarian cancer. Intensive research in BRCA1 has revealed little about its specific role in cancer; rather, this protein has been implicated in a multitude of important cellular processes. The diverse biochemical activities of BRCA1 combine to protect the genome from damage. New data reveal that BRCA1 transcriptionally regulates some DNA-repair genes, and, in addition, new roles for BRCA1 have been identified in heterochromatin formation on the X chromosome, double-strand-break repair, and ubiquitination. These diverse activities of BRCA1 may be linked in a single pathway, or BRCA1 might function in multiple nuclear processes.

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Abbreviations

BARD1	BRCA1-associated RING domain protein 1
BRCA1	breast cancer susceptibility gene 1
BRCT	BRCA1 carboxy-terminal repeat
holo-pol	RNA polymerase II holoenzyme
NHEJ	nonhomologous end-joining

Introduction

When the BRCA1 tumour suppressor is mutated in breast or ovarian cells, tumours arise, but mutation of BRCA1 in other cell types is lethal. This BRCA1 conundrum highlights two questions: what is the required function of the BRCA1 protein in all cell types? And what is different about the activity of BRCA1 in breast and ovarian cells?

Puzzlingly ubiquitous, BRCA1 functions in the nuclear processes of transcription, chromatin remodeling and silencing, and in various DNA repair mechanisms. It also has ubiquitin ligase activity. Creating further confusion, the number of proteins that interact with BRCA1 is astronomical. In this review, we will outline the known nuclear functions of BRCA1, and we will attempt to integrate these seemingly disparate functions into a single pathway. The even more perplexing extranuclear functions of BRCA1, including regulation of centrosome replication and cytokinesis [1–4], will not be discussed in this review.

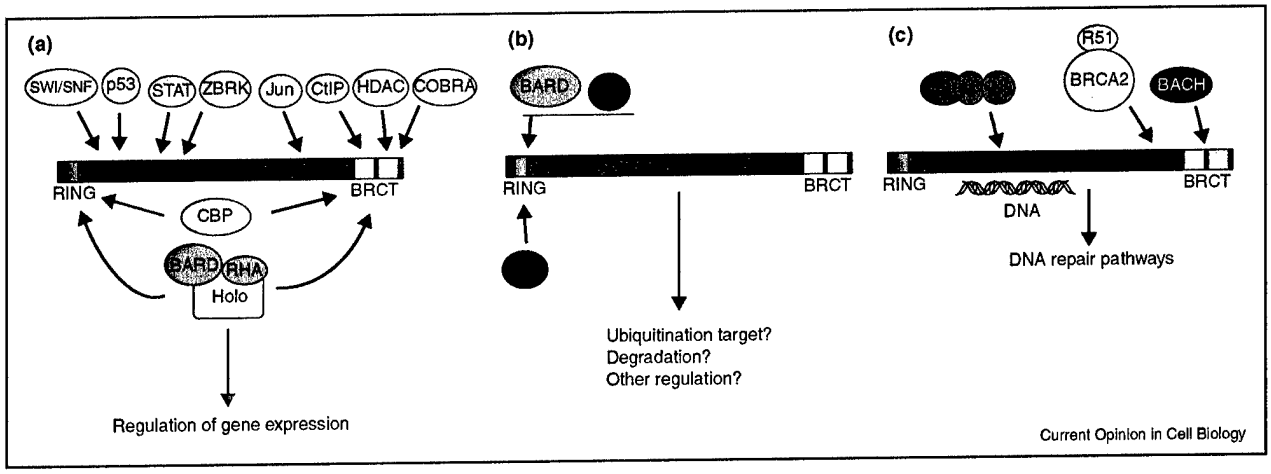
BRCA1 and transcription

The concept of BRCA1 as a transcription factor was first suggested by transient transfection assays in which a reporter gene was activated by the carboxyl terminus of BRCA1 fused to the GAL4 DNA-binding domain [5,6]. Because this assay can yield a positive result for non-transcription factors, the transcription function of BRCA1 was established by the association of BRCA1 with RNA polymerase II in a large complex called the RNA polymerase II holoenzyme (holo-pol) [7]. BRCA1 was also shown to regulate transcription in a purified *in vitro* system [8]. BRCA1 does not fit the model of an enhancer-binding protein, since it does not bind to DNA with sequence-specificity. BRCA1 does, however, bind to DNA independently of sequence, but with a preference for abnormal structure [9], but this is more consistent with a role in DNA repair rather than transcription.

BRCA1 associates with the holo-pol via its extreme ends: the amino-terminal RING-finger domain provides the primary binding to the holo-pol, probably via its association with BARD1 (BRCA1-associated RING domain protein 1), which is also a component of the holo-pol [10•]. The carboxyl terminus of BRCA1 also binds to the holo-pol via its association with RNA helicase A [11]. The internal portion of BRCA1 binds to a large number of enhancer-binding factors (a subset of interacting transcription factors are shown in Figure 1), and most transient transfection results are consistent with a co-activator function in which BRCA1 bridges the specific enhancer-binding factor to the holo-pol complex.

Finding target genes regulated by BRCA1 overexpression in tissue culture might give insight into genetic pathways abnormally expressed in cells that have mutated BRCA1. Several such experiments have been done using microarray technology [12–14,15••,16•,17]. p53-responsive cell cycle progression inhibitor and stress-response factors such as p21 and GADD45, respectively, are stimulated by BRCA1 overexpression, and BRCA1 has been shown to be a co-activator for p53 [12,18–20]. In a recent study [15••], p53 was stabilized by overexpression of BRCA1; however, unlike stabilization of p53 by DNA damage, BRCA1 stimulated those p53 pathways that lead to survival and repair, rather than to apoptosis. For example, BRCA1 synergistically stimulated cell cycle arrest and

Figure 1



A subset of known nuclear interactions by BRCA1 are shown and grouped according to function: **(a)** Transcription and chromatin remodeling, **(b)** ubiquitination, and **(c)** DNA repair. The approximate positions of the interaction sites along the BRCA1 polypeptide are indicated by arrows. Some of the interactions shown are not specifically discussed in this review, but described in other reviews [54]. BARD, BARD1; CBP, CREB-binding protein; COBRA, cofactor of BRCA1; CtIP, carboxy-terminal binding protein interacting protein; HDAC, histone deacetylase; R51, RAD51; RHA, RNA helicase A; R-M-N, RAD50–MRE11–NBS1 complex; ZBRK, zinc-finger- and BRCA1-interacting protein with a KRAB domain.

DNA-damage repair genes normally upregulated by p53 (such as *p21* and *p53R2*). By contrast, *PIG3*, *PERP* and *Bax*, genes involved in p53-dependent apoptosis, were downregulated by BRCA1 overexpression [15••]. Knocking down BRCA1 expression using antisense RNA led to an increased sensitivity of cells to apoptosis [15••]. Interestingly, BRCA1 does not always save cells from imminent death–overexpression of BRCA1 leads to the up-regulation of genes necessary for apoptosis via the interferon-γ pathway [16•].

In the near future, similar microarray studies, using knockdown of BRCA1 by small interfering RNA, might be able to identify genes with expression affected by loss of BRCA1, mirroring that which occurs in BRCA1-associated cancer. In these studies, it will be important to identify genes in non-breast cells whose loss of function might result in cell death, and also other genes in normal mammary cell lines whose loss of function results in unrestrained growth.

BRCA1 and chromatin remodeling

BRCA1 has a multifaceted role in transcription. It regulates many types of genes, interacts with transcriptional repressors and activators, and it is also part of the transcriptional machinery. One way to link these roles is to hypothesize that BRCA1 controls chromatin structure. It has been shown to interact with the chromatin remodeling factors SWI/SNF and BRG1 [21,22] and with histone deacetylase [23] (Figure 1a). Recent functional assays showed that full-length BRCA1 tethered to heterochromatin resulted in a partial decompression of the DNA [24••]. In this assay, the fragment of BRCA1 containing the carboxy-terminal BRCT (BRCA1 carboxy-terminal repeat) domain vastly reversed the compression of the heterochromatin. Since isolation of the BRCT domain reveals a hyperactive protein, it is suggested that this remodeling function is negatively regulated by the rest of the BRCA1 protein [24••]. Although these experiments clearly demonstrate that BRCA1 has a role in regulating the maintenance of heterochromatin, the nature of the experiment tethers high concentrations of protein on a megabase heterochromatin domain, and the decompression by BRCA1 might be specific for the experimental system.

The flip side of this last finding was observed for a role in the establishment of heterochromatin by BRCA1. Mutation, or loss of function, of BRCA1 results in an altered phenotype of X chromosome inactivation [25••], a process by which a major heterochromatin domain is established over one X chromosome. *XIST* is an RNA molecule that coats the inactive X chromosome in female cells and is central to the process by which the entire chromosome is repressed (see the review by Andersen and Panning, this issue). When BRCA1 is not present in a cell, *XIST* RNA fails to localize to the X chromosome. The presence or absence of functional BRCA1 does not affect the level of the *XIST* transcript, just its localization and effectiveness in silencing. Other heterochromatin markers such as macro-histone H2A1 and H3 methylated at Lys9, also failed to localize to the inactive X chromosome without functional BRCA1 [25••]. This intriguing finding leaves unanswered how this *XIST* RNA localization phenotype might promote breast cancer. Perhaps the de-repressed

X chromosome expresses an oncogene at higher levels than in cells that have one inactivated X chromosome.

BRCA1 and DNA damage repair

BRCA1 co-localizes with macro-H2A1 and H3mK9 (histone H3 methylated at Lys9) at the inactive X; it has also been found to co-localize with another modified histone, phosphorylated H2AX (γ-H2AX), after DNA damage [26]. Unphosphorylated H2AX is interspersed in chromatin throughout the genome, and following DNA damage, one of the earliest events is the phosphorylation of Ser139 of H2AX in large DNA domains encompassing a million base pairs [27]. γ-H2AX forms discrete foci within 10 minutes of DNA damage with BRCA1 detectable in these foci as fast as within 30 min in some cell lines, as slow as six hours in others. After BRCA1, either RAD50 or RAD51, but not both, also co-localize with DNA-damage-induced foci [26].

Although these foci are assembled at sites of DNA damage, the slowness of formation is inconsistent with the timing of the bulk of the repair process [28]. Not surprisingly, H2AX-null cells are hypersensitive to ionizing radiation and have increased spontaneous chromosomal aberrations. Formation of BRCA1 and RAD51 DNA-damage-induced foci were decreased [29•,30•], suggesting that γ-H2AX lies upstream of microscopically detectable association of BRCA1 with these damage sites. Interestingly, tethering of BRCA1 to the chromosome caused γ-H2AX phosphorylation to co-localize with the BRCA1 without DNA damage. BRCA1 might therefore recruit the kinase that phosphorylates H2AX to the DNA and nucleate a repair focus [24••]. Taken together, it appears likely that BRCA1 at low concentrations recruits the H2AX kinase, creating a repair domain, which then assembles the foci that eventually accumulate high concentrations of BRCA1.

Besides these curious repair foci, BRCA1 regulates a variety of DNA-damage-repair pathways. BRCA1-deficient cells have defects in transcription-coupled repair, homologous recombination, nonhomologous end-joining (NHEJ), and microhomology end-joining [31–34, 35••, 36]. *In vitro* binding assays have also revealed that BRCA1 binds to several factors involved in the repair pathways listed above (Figure 1c). The diversity of the repair mechanisms is suggestive of an indirect effect of BRCA1 on these various pathways. BRCA1 could transcriptionally activate the genes encoding the repair-effector enzymes (see above), or, alternatively, some other activity such as ubiquitination (see below) might regulate the effector proteins. However, in one case a clear biochemical requirement for normal BRCA1 function was established in a cell-free NHEJ assay [35••], which is likely to be dependent on BRCA1 association with the complex containing the RAD50–MRE11–NBS1 complex [37,38].

BRCA1 and ubiquitination

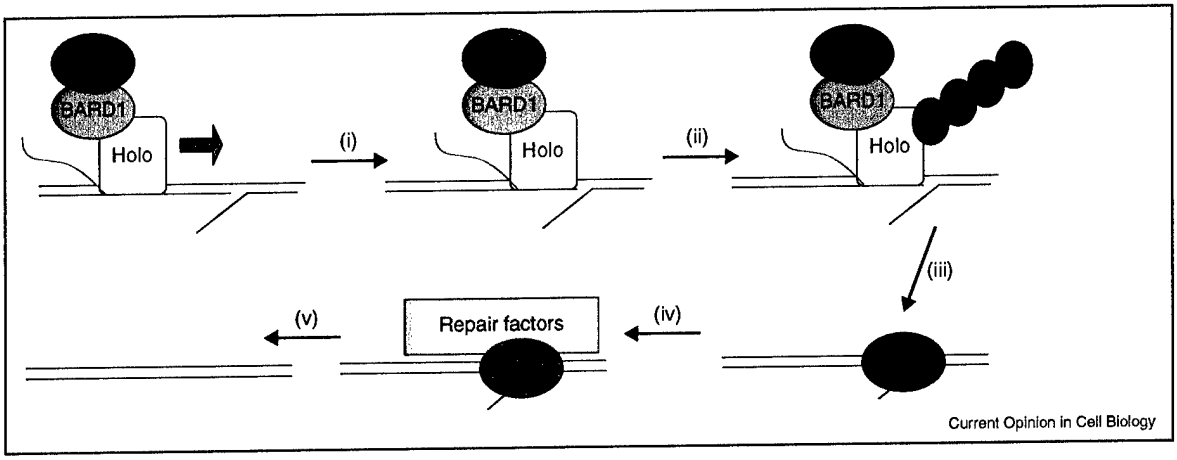
The RING-finger domain, such as is found at the BRCA1 amino terminus, is commonly associated with ubiquitin ligase activity. A ubiquitin ligase polymerizes ubiquitin on a target protein. That ubiquitinated target is then degraded by the proteasome. This process is quite specific, as individual polypeptides in a protein complex may be ubiquitinated and degraded, leaving behind the other subunits intact [39]. BRCA1, together with BARD1, form a heterodimer associating via the RING-finger domains and adjacent α helices, and BRCA1–BARD1 is an active ubiquitin polymerase (Figure 1b) [40,41]. A ubiquitin polymerase will synthesize long chains of ubiquitin, but the key will be to identify the target for the ubiquitin ligase function. Although ubiquitination is usually linked to protein degradation via the 26S proteasome, BRCA1–BARD1 might also monoubiquitinate proteins, or polymerize ubiquitin via different lysine linkages that do not target the protein to degradation but into some other pathway. BRCA1–BARD1 monoubiquitinates histone monomers, including unphosphorylated H2AX [42,43,44•]. It will be important to test whether BRCA1–BARD1 ubiquitinates histones in octamers on DNA, and also H2AX in a phosphorylation-specific manner.

Most RING-finger proteins do not specify the ubiquitination target via the RING-finger domain, but rather by some other domain of the same polypeptide, or another subunit of multisubunit ubiquitin ligases. All but one of the current studies use isolated BRCA1–BARD1 RING-finger domains. BRCA1–BARD1 are large proteins and thus it is likely that the untested 90% of these proteins regulate the ubiquitin ligase enzymatic activity or specify its target. Ideally, using full-length BRCA1–BARD1, specific targets of ubiquitination and functional consequences will be identified in the near future.

The RING fingers of BRCA1 and BARD1, which ordinarily heterodimerize, can form 30 nm ring-shaped superstructures that are visible by electron microscopy. These super-RING structures are more enzymatically active than dimeric BRCA1–BARD1, and the ring structure functions as a scaffold for other proteins to couple reactions. The E2 ubiquitin conjugating enzyme, UbcH5c, forms a circle surrounding the BRCA1–BARD1 complex, and ubiquitin chains dot the surface. This scaffold arrangement renders the ubiquitination activity of BRCA1–BARD1 highly processive [45••].

A potential link of this ubiquitination activity to the transcription function of BRCA1 could be via the recently established role of ubiquitination in transcriptional activation [46]. BRCA1 may ubiquitinate the enhancer-binding factors it co-activates. Alternatively, transcriptional silencing has been observed to be mediated in part via monoubiquitination of histones.

Figure 2



A model is outlined, which links the multiple biochemical activities of BRCA1 into a genome maintenance pathway. BRCA1, along with an elongating polymerase, would function in the surveillance for DNA damage. Once a damage site is encountered (step 1), BRCA1 would ubiquitinate the holo-pol, marking it for destruction (step 2), BRCA1 remains bound to the DNA at the lesion (step 3) to recruit the repair factors (step 4), and the lesion is repaired (step 5).

An integrated model for BRCA1 function in genome stability

BRCA1 functions in several processes, but it is unclear how these relate to the BRCA1 requirement in all cell types. Similar to the p53 tumour suppressor, BRCA1 activates genes encoding the DNA-repair response. Unlike p53, BRCA1 also has a direct role in the repair process. We update here a model suggested earlier [47]. According to this model, BRCA1–BARD1 functions in genome surveillance by scanning active genes in association with the holo-pol, and when the elongating transcription complex encounters DNA lesions, BRCA1 initiates a repair response (Figure 2). It is interesting to note that a BRCA1-binding cofactor, COBRA1, which regulates BRCA1 function in a chromatin decompression assay [24••], has been found to be a required subunit of a complex that regulates transcription elongation [48]. When damage is encountered on the DNA template, the lesion could be corrected by transcription-coupled repair (step 1), a known BRCA1 function [31,32]. Alternatively, some types of damage might require that the polymerase be removed to effect repair. Since the polymerase synthesizing mRNA on a DNA template is quite stably bound [49], we hypothesize that BRCA1 would then ubiquitinate the polymerase signaling its degradation (step 2). Although current evidence does not implicate BRCA1 in this process, the polymerase is ubiquitinated and degraded following DNA damage [50,51••]. The residual BRCA1 complex might remain bound to the DNA lesion. BRCA1 has been found to bind DNA cruciforms and three-way junctions, such as might occur at damage sites [9] (step 3). This bound BRCA1 would then recruit repair factors, such as the RAD50-containing complex, which would then mend the lesion (steps 4 and 5). One might infer from the recruitment of the H2AX kinase to sites in which BRCA1 is bound to DNA [24••] that this surveillance of the template by transcription results in BRCA1-dependent degradation of the transcription apparatus and recruitment of the H2AX kinase to nucleate the assembly of a repair focus. Although there is no yeast homolog for BRCA1, perhaps a analogous pathway is conserved in this organism, mediated by a transcription-elongation factor that is genetically linked in this pathway to holo-pol components [52,53].

Conclusions

Currently, the key cellular functions assigned to BRCA1 are uncomfortably numerous. BRCA1 can interact with many cellular proteins and pathways, but how these many interactions address the key questions of required ubiquitous function and tumour suppressing breast and ovarian cell function are unclear. We suggest that several of these pathways can be assimilated into a single pathway, which controls genomic stability in all cell types.

Acknowledgements

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The domains required for BRCA1 association with the holo-pol were determined, and deletion of the amino-terminal domain resulted in a reduction of BRCA1 association with the RNA polymerase II holoenzyme (holo-pol) by 98%. Because BARD1 was also found associated with the holo-pol, this interaction of BRCA1 with holo-pol might occur via BARD1. The authors also showed that the amino-terminal domain of BRCA1 is essential for association of BRCA1 into foci in S phase of the cell cycle. The BRCT domain had a minor contribution of BRCA1 association with the holo-pol.

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